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Staphylococcus Epidermidis Adhesion to Modified Polycarbonate Surfaces: Gold and SAMs Coated

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Abstract

Bacterial adhesion to a biomaterial surface is thought to be the key step in the infection of indwelling medical devices and constitutes an object of study in the demand to reduce nosocomial infections. In this work, specific modifications on polycarbonate outer layer were utilized as model surfaces for the study of the adhesion of *Staphylococcus epidermidis*, one of the main microorganisms responsible for nosocomial infections. The effect of gold coating on staphylococcal adhesion was assessed, as well as of subsequent coverage with different self-assembled monolayers (SAMs): two SAMs with a methyl terminal group and hydrophobic character and two hydrophilic SAMs with a carboxylic acid terminal group. Variations in the aliphatic chain length were also tested. A SAM with a calix-crown molecule was also created to immobilize a specific protein and its antibody. The extent of staphylococcal adhesion to methyl terminated SAMS was reduced compared to the number of cells adhered to the carboxyl acid terminated SAMs, demonstrating that methyl terminated SAMs constituted more suitable surfaces in preventing bacterial adhesion. The calix-crown molecule favours high levels of adhesion due to its non-specific bonding nature and geometrical configuration. However, when a specific protein is linked to calix-crown, bacterial adhesion occurs to a much lower extent. The results obtained in this work have a potential practical significance showing that the use of certain SAMs as surface modifiers may constitute a successful method in the reduction of bacterial adhesion to biomedical surfaces.

Keywords

Adhesion, calix-crown, polycarbonate, self-assembled monolayers, *Staphylococcus epidermidis*

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1. Introduction

Microbial adhesion to solid surfaces, in aqueous medium, is ubiquitous and is the prime step in biofilm formation. *Staphylococcus epidermidis* is a coagulase-negative staphylococcus (CNS) that is widely distributed over the surface of the human body, where it constitutes, together with other CNS, the majority of the commensal bacterial microflora [1, 2]. However, these staphylococci have emerged in the last years as the most frequently isolated pathogens in nosocomial sepsis and currently cause more infections associated with the use of medical devices, such as catheters, than any other group of microorganisms [3, 4]. This is due to the ability of CNS to adhere and to form biofilms on biomaterial surfaces, making the microorganism more resistant to antibiotics and to the host defence mechanisms [3, 5]. Therefore, initial adhesion of bacteria to the biomaterial surface is thought to be the key step in the infection of indwelling medical devices [6] and constitutes a challenge to the development of less adherent surfaces.

In the last decades intensive research has been carried out in order to determine the parameters which control the interaction between bacterial cells and surfaces. Many surface properties have been shown to play an important role, such as hydrophobicity [7, 8], electrical charge [9], the presence of proteins [10, 11] and surface chemistry [12, 13]. A rigorous study of the effect of these properties on the adhesion process requires a model system that allows precise control of the type and the configuration of functional groups of the substrate surface [14]. The advent of self-assembled monolayers (SAMs) has provided a way to fabricate well-defined model surfaces of known structures and properties that can be regulated and manipulated. The terminal group of the precursor molecules from which SAMs are formed determines the surface chemistry. In general, the preparation of SAMs consists in the deposition of a solution of the molecule of interest onto the substrate surface and a final wash to remove the excess material. SAMs formed by the adsorption of ω-substituded alkanethiols on gold, and to a lesser extent alkyltrichlorosilanes on hydroxylated surfaces, are the most common and most highly ordered surfaces currently available for studying the interaction of cells and proteins with substrata of different surface chemistry [14]. This is because sulphur has a very good affinity for gold and the alkanes with a thiol head group bond to gold, outlining an ordered assembly with a dense packing of alkyl chains due to van der Waals forces. Prime and Whitesides [15] consider that SAMs of alkanethiols on gold are useful model systems for investigating mechanisms of protein adsorption. Wiencek and Fletcher [16] also used methyl and hydroxyl terminated SAMs for the study of the effect of surface free energy on bacterial adhesion. More recently, Lee *et al*. [17] used SAMs with different functional groups (X=OH, COOH, NH_2 and CH_3) to study the effect of the physicochemical characteristics of a material surface on the $\alpha 5\beta 1$ integrin-mediated adhesion of a cell line of erythroleukemia cells to fibronectin, one of the physiological fluid proteins that adsorbs onto the biomaterial surface immediately after exposure to it. Wang and co-workers [18] also used a cell line of bovine aortic endothelial cells and SAMs of alkanethiolates to study

how surface parameters, such as surface charge, affected the structure and activity of adsorbed proteins and, consequently, cell adhesion.

Besides the terminal group, it has been reported that the alkyl chain length of a SAM also exerts a significant influence on its behaviour [13] such as, for example, the variation of its wettability according to the chain length [19, 20]. The wettability of a SAM can also be influenced by the van der Waals forces of the metallic substrates, such as gold, that support the SAM [21].

The aim of the present work was to use modified polycarbonate surfaces to assess *S. epidermidis* adhesion ability. The polycarbonate surface alterations included coating of polycarbonate surface with gold and its subsequent coverage with hydrophobic and hydrophilic SAMs, both with variations in the aliphatic chain length. A calix-crown SAM was also created to immobilize a specific protein and its antibody, for evaluation in terms of bacterial adhesion as well.

2. Material and Methods

2.1. Bacteria and Growth Conditions

The strain used in this work was *S. epidermidis* 9142, a clinical and biofilm positive strain provided by Gerald B. Pier, Harvard Medical School, Boston, USA. Trypticase soy broth (TSB) and trypticase soy agar (TSA) plates were prepared according to the manufacturer's instructions. The strain was grown for 24 (±2) h at 37°C in a shaker rotating at 130 rpm in 15 ml of TSB using as inoculum bacteria grown on TSA plates not older than 2 days. Then, 50 µl of cell suspension were transferred to 30 ml of fresh TSB and incubated for 18 (±2) h at 37°C and 130 rpm. After being harvested by centrifugation (for 5 min at 9000*g* and 4°C), cells were resuspended in saline solution (0.9% NaCl prepared in distilled water) followed by centrifugation (5 min at 9000*g* and 4°C) and this procedure was repeated twice. Finally, they were resuspended in saline solution at a concentration of approximately 1×10^9 cells/ml, determined by optical density at 640 nm. These cell suspensions were used in the subsequent adhesion assays.

2.2. Substrate Preparation

The polycarbonate substrate (PC) was cut into 2.0 cm × 2.5 cm coupons and the gold film (G) was deposited by ion sputtering (Sputter Coater SC502, Fisons Instruments, UK) to a thickness of approximately 75 nm. At the same time, solutions of octanethiol [$HS(CH_2)7CH_3$] (OT), hexadecanethiol [$HS(CH_2)15CH_3$] (HDT), mercaptoacetic acid [$HSCH_2COH$] (MAA), mercaptopropionic acid [$HS(CH_2)2CO_2H$] (MPA) and calix-crown (CC) were prepared in Petri dishes, at a ratio (wt/wt) of 99.9% ethanol to 0.1% of the respective compound. The gold covered polycarbonate samples were washed in ultra-pure water, dried and placed in the respective solutions. The Petri dishes were sealed with parafilm, left overnight at 25°C and protected from light. Next, only the coupons with the calix-crown monolayer were immersed for 1 h in a phosphate buffered saline (PBS) solution

(pH 7.4), with a monoclonal antibody, anti-C-Reactive Protein (CRP) (100 μg/ml). Following this step, coupons were washed again with ultra-pure water (Millipore Direct-Q, 18 MΩ/cm resistivity) and immersed, for 1 h, in a PBS solution (pH 7.4) with the respective anti-gene, CRP (100 μg/ml).

2.3. Bacteria and Substrata Hydrophobicity

Hydrophobicity of bacteria and substrata was evaluated through water contact angle measurements. For contact angle measurements on substrata, a micropipette was adapted to a module enabling movement along the vertical and horizontal axes, connected to an optical microscope, in order to deposit a liquid drop (2 μl) onto the coupon, which was in a saturated atmosphere chamber. The drop image was captured by an optical system consisting of a CCD (Charge-Coupled Device) video camera (640 × 480 pixels), with amplification lenses. In the case of bacteria, the measurements were performed on bacterial layers deposited on membrane filters, according to the method described by Busscher *et al.* [22]. Contact angle measurements (at least 25 determinations) were done using the sessile drop technique on the cell lawns, using a contact angle measurement apparatus (model OCA 15 PLUS, DATAPHYSICS). All measurements were performed at room temperature, with ultrapure water with an electrical resistivity of 17.8 MΩ/cm (Nanopure, Barnstead).

2.4. Initial Bacterial Adhesion

Coupons of the substrata were placed in 6-well tissue-culture plates containing 4 ml of bacterial suspension (1×10^9 cells/ml) in saline solution. Initial adhesion to each substrate was allowed to occur for 2 h at 37°C, in a shaker rotating at 130 rpm. Negative controls were obtained by placing the coupons in a saline solution without bacterial cells. The coupons were then gently transferred to a 100 ml glass beaker containing distilled water, and were allowed to rest there for approximately 10 s. Afterwards, a new transfer was made to a different 100 ml glass beaker containing distilled water, followed by a third transfer 10 s later. These washing steps were carefully performed in order to remove loosely attached cells [23]. The substrate coupons with adhered cells were dried at 37°C. All experiments were done in triplicate and repeated in four independent assays.

2.5. Image Analysis

Before imaging and enumeration of adhered cells, the substrate coupons were stained with a 0.01% DAPI (4′-6-diamidino-2-phenylindole) solution, for better image contrast. After 30 min, each coupon was rinsed with distilled water in order to remove the excess stain, left to air-dry and kept in the dark.

Direct bacterial counts were done using an epifluorescence microscope (Carl Zeiss, Germany) with a filter sensitive to DAPI fluorescence and coupled to a 3 CCD video camera that acquires images with 820 × 580 pixels resolution and at a magnification of 1000×. Enumeration of adhered cells was performed with an automated enumeration software (SigmaScan Pro 5) and the results were presented as number of adhered cells/cm^2.

2.6. Statistical Analysis

The resulting data were analysed using the Statistical Package for the Social Sciences Software (SPSS, Inc., Chicago). The comparison was performed through one-way analysis of variance (ANOVA) by applying the Bonferroni analysis as a post hoc test. All tests were performed with a confidence level of 95%.

3. Results

3.1. Surface Characterization

Considering that SAMs were prepared on gold films supported on polycarbonate surfaces, the polycarbonate (PC) and gold-coated (G) surfaces were also tested as substrata and used as control surfaces.

The values of water contact angles (°) on the different substrata assayed are presented in Table 1. According to Vogler [24], hydrophobic surfaces exhibit water contact angle values higher than 65°, whereas hydrophilic ones exhibit water contact angles lower than 65°. Thus, PC, G, OT, HDT and CC surfaces are hydrophobic, while MAA, MPA and CRP surfaces are hydrophilic. However, it must be noted that the three last ones are only slightly hydrophilic, with an almost hydrophobic character. They can be considered moderately wettable surfaces.

3.2. Cell Adhesion to Substrata

A crucial factor for the preparation of high-quality SAMs is the surface condition of the gold-coated substrate, because this metal is easily contaminated by the carbonaceous contaminants present in the atmosphere [25]. *S. epidermidis* adhered to the gold control surface and to the OT SAM in a lower extent compared to all the other surfaces (Figs 1 and 3). On the other hand, the number of bacterial cells adhered to PC was slightly higher when compared with that adhered to HDT and MAA SAMs and statistically higher ($p < 0.05$) in comparison to G and OT

Table 1.
Water contact angles (°) of the materials and the *S. epidermidis* strain

	Water contact angle ± SD
S. epidermidis 9142	25.6 ± 0.9
PC	87.2 ± 3.4
G	87.5 ± 2.0
OT	96.7 ± 4.6
HDT	101.9 ± 0.5
MAA	59.6 ± 3.8
MPA	53.8 ± 1.7
CC	85.0 ± 1.2
CRP	45.8 ± 1.1

Results are presented as mean ± standard deviation (SD).

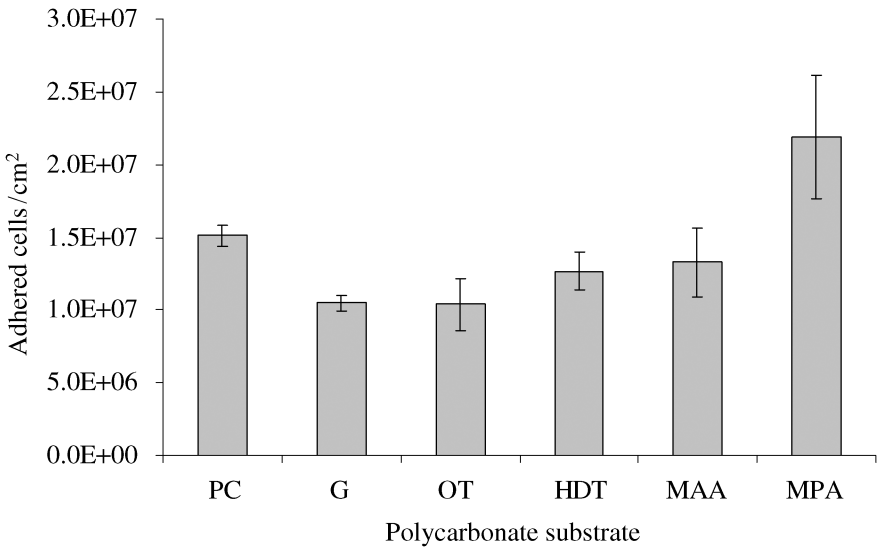

Figure 1. Number of *S. epidermidis* cells adhered per cm^2 on PC (polycarbonate) coupons and on modified polycarbonate surfaces: G (gold), OT (octanethiol), HDT (hexadecanethiol), MAA (mercaptoacetic acid) and MPA (mercaptopropionic acid).

SAMs. Analysing the adhesion values of the two methyl (CH_3) (OT and HDT) and carboxylic acid (COOH) terminated SAMs (MAA and MPA) it can be seen that, in the latter, cells adhered to a higher extent than to OT or HDT (Figs 1 and 3). However, this difference was only statistically significant ($p < 0.05$) in the case of MPA. The results of the present study are in agreement with other literature reports [13, 26, 27], where high levels of cellular attachment were observed to carboxylic acid terminated SAMs as opposed to low levels of attached cells to methyl terminated SAMs. Regarding the effect of the aliphatic chain length on bacterial adhesion to hydrophobic SAMs (OT and HDT), the present results might suggest that an increase in chain length promotes a slight increase in the number of adhered cells (Fig. 1). However, no statistical significance ($p > 0.05$) was found. Conversely, in the case of hydrophilic layers (MAA and MPA), a slight increase in the alkyl chain length promotes a substantial increment in the number of adhered cells.

As for the calix-crown SAM, it enables a non-specific immobilization of protein/antibodies and, as a result, it should favour bacterial adhesion due to its non-specific protein binding nature. Thus, the extremely high levels of *S. epidermidis* adhered to the CC SAM were expected. The number of cells attached to the complex anti-CRP — CRP immobilized by the calix-crown was approximately double of that to the gold surface. However, *S. epidermidis* cells adhered to a much lower extent to the anti-CRP — CRP substrate when compared to the number of cells adhered to the CC SAM (Figs 2 and 3).

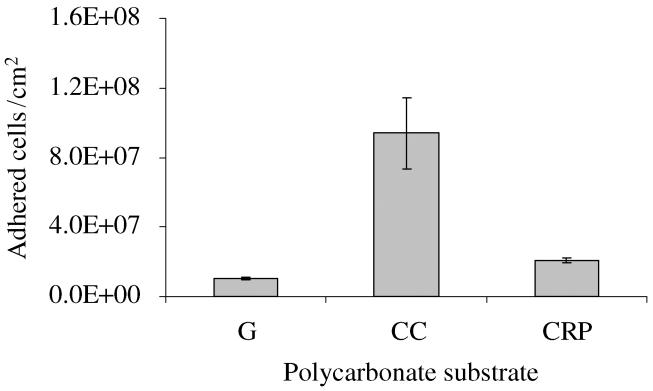

Figure 2. Number of *S. epidermidis* cells adhered per cm^2 on modified polycarbonate surfaces: G (gold), CC (calix-crown) and CRP (calix-crown with the antibody anti-CRP and the respective anti-gene, CRP).

4. Discussion

Adhesion of bacteria to a given surface can be influenced by several factors such as hydrophobicity, the nature of the material and the immobilization of proteins on the surface. However, even very subtle changes in the surface layer structure are also important, with the terminal group and the length of the alkyl chain playing a significant role in bacterial adhesion. According to Arima and Iwata [28], one of the factors that mostly affects cell adhesion is the wettability of the substrate surface and it has been considered that wettability of SAMs varies with chain length [19, 20]. Faucheux *et al*. [29] found that SAMs terminated with CH_3 produced hydrophobic surfaces (water contact angle $> 80°$), while those with –COOH formed moderately wettable surfaces (water contact angle between 48–62°), which is in agreement with the results obtained in the present work (Table 1). According to the study of Lee *et al*. [30] the maximum cell adhesion to polyethylene surfaces with a wettability gradient was found for a water contact angle of 55°, which is very close to the MPA water contact angle value determined in this study ($53.8 \pm 1.7°$). It must be noted that on MPA SAM (with the exception of CC SAM) *S. epidermidis* adhered to the highest extent. In fact, some authors report that cell adhesion to polymer surfaces is favoured with moderate wettability and water contact angle between 40–70° [31, 32].

The water contact angle determined here for PC surface is very similar to the value determined by Rios *et al*. ($81.3 \pm 0.7°$) [33]. Also, the values of water contact angles determined on the gold and methyl-terminated surfaces are close to the ones found in the literature where the values varied between 71–83° and 108–116° for gold and methyl terminated surfaces, respectively [13, 25, 34–36]. However, in the case of the carboxylic acid terminated SAMs, the water contact angle was higher than most of the literature reported values: Scotchford and co-workers [36] as well as Cooper *et al*. [13] obtained water contact angle on MPA less than 15°, while

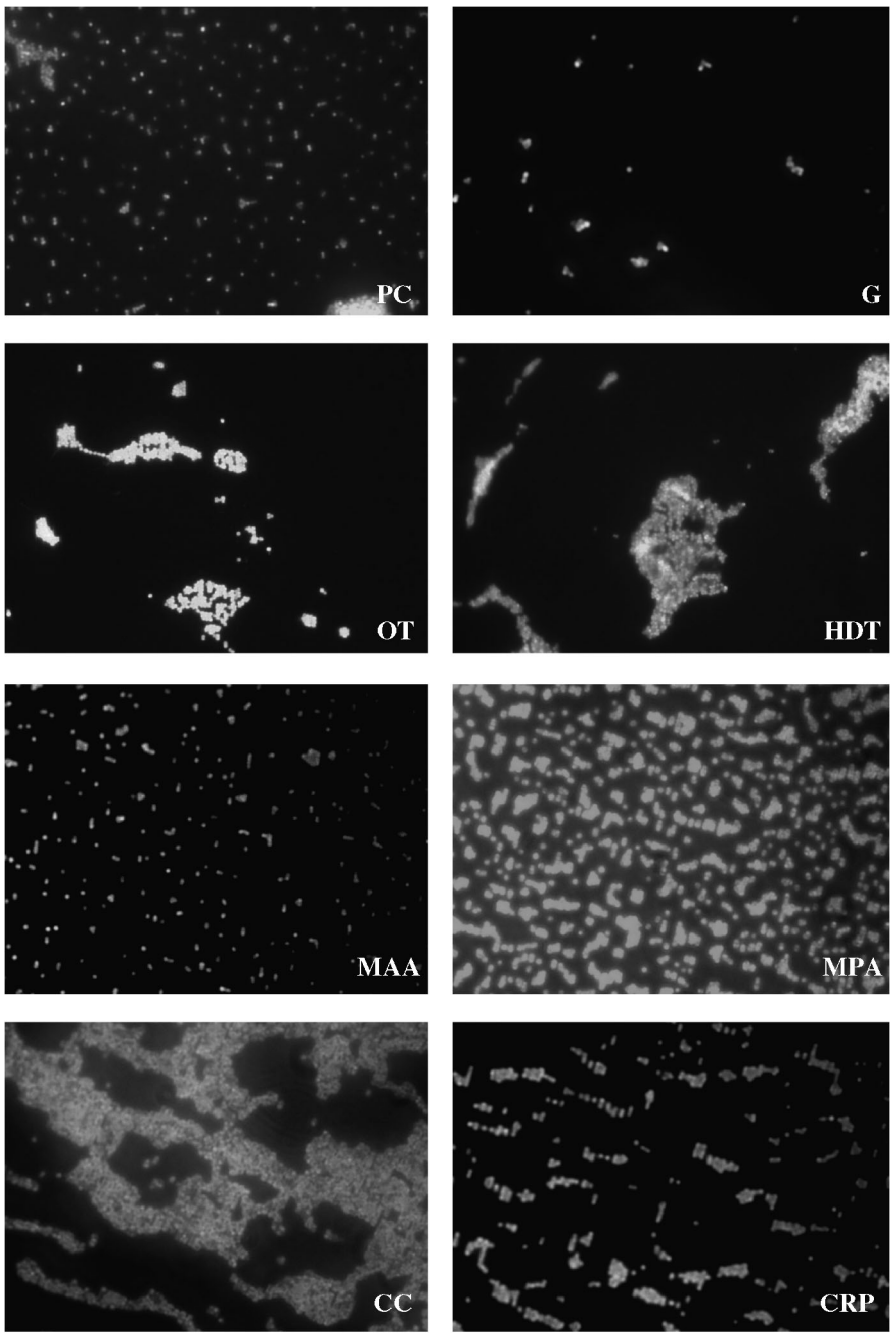

Figure 3. Representative epifluorescence microscopy photographs of the extent of adhesion of *S. epidermidis* cells to the PC (polycarbonate) coupons and to the modified polycarbonate surfaces: G (gold), OT (octanethiol), HDT (hexadecanethiol), MAA (mercaptoacetic acid), MPA (mercaptopropionic acid), CC (calix-crown) and CRP (calix-crown with the antibody anti-CRP and the anti-gene, CRP).

Tsai and Lin [25] and Lin and Chuang [35] obtained for the COOH SAMs the values of 4.3° and 33.5°, respectively. These differences were probably due to the highly polar nature of the carboxylic group as well as to its predisposition to rapidly adsorb laboratory contaminants [14]. Another important point to stress is that, in this study, the gold coating was made on polycarbonate substrate while the majority of the studies available in the literature are about SAMs deposited on gold films on coverglass or silicon wafers, with different surface roughness/topography. Such differences might have led to variations in the surface characteristics of the SAMs.

The modification of the polycarbonate surface with gold and with SAMs lowered the levels of *S. epidermidis* adhesion in all four of the studied situations: simply by coating with gold and by layering with OT, HDT and MAA SAMs, although the last two ones had a less significant effect. The gold layer constitutes a high-quality support for the adsorption of the different SAMs, forming an extremely ordered and stable monolayer with a rigorous control on the terminal group functionality and surface chemistry [13]. However, the present results show that the gold layer *per se* is very effective in lowering microbial adhesion. In fact, the antimicrobial effects of gold and its use in medicine are already well known [37] and once again are corroborated by the present work.

As regards the behaviour of the terminal group, the results of the present study are in agreement with the values of the extent of adhesion described in the literature, despite the difference in the nature of the adherent specimens. Lin and Chuang [35] observed that platelets adhered in much higher extent to a COOH SAM than to CH_3 terminated SAM. Also Tsai and Lin [25] showed that the platelet adhesion intensity increased in the following order: gold $<$ CH_3 $<$ COOH terminated SAMs. According to the work of Scotchford *et al*. [36] osteoblasts adhered preferentially to COOH and then to CH_3. Faucheux *et al*. [29] demonstrated that after a 2-h period, fibroblasts adhered more to SAMs with COOH terminal group than to SAMs terminated with CH_3. Nevertheless, it is important to note that a large amount of the available adhesion studies to SAMs concern different cell types which display distinctive responses to a particular surface. The behaviour and the mechanism by which an animal tissue cell adheres to a specific surface may be very different from the one of a *S. epidermidis* cell. Regarding the effect of the alkyl chain length on the adhesion of *S. epidermidis* cells, no effect was detectable in methyl terminated SAMs, while on carboxylic SAMs, a subtle increase in chain length from MAA to MPA seems to exert some influence, with MPA exhibiting higher levels of adhesion in comparison to MAA. Hence, it becomes difficult to understand which one of these factors has the strongest influence in *S. epidermidis* adhesion. In this particular case, it seems that the nature of the terminal group in combination with a moderate wettability exerts a critical effect in *S. epidermidis* adhesion.

Calix-crown SAM is a particular case, due to its conformation and its non-specific protein binding nature. Cell wall proteins of the bacteria, such as adhesins, have a perfect niche for binding, so the adhesion is extremely enhanced. In this case, cell proteins bind in a non-specific way to the calix-crown molecule, and without

competition with other proteins. This molecule can also be used as an artificial linker system for protein immobilization [38] by pre-binding a specific antibody to promote the selective linkage of a given protein. This is very important because protein adsorption onto the surface of a foreign material is the early event for a set of varied applications such as biosensors, biochips, bioreactors and diagnostic techniques [39]. In this work, the C-reactive protein (CRP), a biomarker for acute levels of inflammation, was immobilized on the calix-crown molecule by means of protein–protein interaction with anti-CRP. In this case, the extent of *S. epidermidis* adhesion was approximately double when compared to the gold-coated surface. In a certain way, this is in agreement with the work of Arima and Iwata [28] where the cellular adhesion was studied both on hydrophobic and hydrophilic SAMs with pre-adsorbed albumin. These authors observed that on hydrophilic SAMs, such as the CRP used here, albumin was replaced by cell adhesive proteins and, as a result, SAMs with moderate wettability became more suitable surfaces for cell adhesion. However, in comparison to the levels of bacterial adhesion to the calix-crown molecule, the values were significantly lower. The CRP and its antibody blocked the sites of bonding of the calix-crown molecule, preventing the linkage of bacterial proteins. This type of SAM may constitute a good alternative to avoid bacterial adhesion to biomaterials. In addition, it is important to stress that under physiological conditions, most of the substrate properties are masked by the presence of an adsorbed protein layer and the effect of substrate properties on bacteria adhesion is minimal [14].

5. Conclusions

The use of SAMs constitutes an excellent method to determine the effect of surface modifications on bacterial adhesion, mainly due to the fact that they are structurally the best-organized and controllable surfaces available. The adhesion of *S. epidermidis* to modified polycarbonate surfaces is strongly determined by parameters such as the nature of the functional terminal group along with surface wettability.

The lowest levels of *S. epidermidis* attachment were observed, in addition to gold covered surfaces, on the methyl terminated SAMs, OT and HDT, demonstrating that methyl terminated SAMs constituted more suitable surfaces in preventing bacterial adhesion. The calix-crown molecule allows high levels of adhesion due to its non-specific binding nature. However, the pre-linkage of a particular protein blocks the sites of adhesion for the cell proteins, resulting in a decrease in the number of attached cells. This can constitute an alternative to control the levels of bacterial adhesion to biomaterials.

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